Special Article

Protein quality as determined by the Digestible Indispensable Amino Acid Score: evaluation of factors underlying the calculation

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The Food and Agriculture Organization of the United Nations recently recommended the adoption of a new and improved scoring system (Digestible Indispensable Amino Acid Score [DIAAS]) to quantify dietary protein quality. The DIAAS is based on the relative digestible content of the indispensable amino acids (IAAs) and the amino acid requirement pattern. Factors involved in calculation of the DIAAS include: use of the content and profile of IAAs as the basis for quality; methods for determination of the protein and amino acid content of the protein source; accuracy of individual requirement values for IAAs; normalization of IAA requirements by the estimated average requirement for protein; and basing the DIAAS on the true ileal digestibility of each IAA in the test protein. This review outlines the rationale for including each of these factors in the calculation of the DIAAS and describes associated potential errors.

INTRODUCTION

Dietary guidelines specify that the requirement for protein should be met through the intake of "high-quality" protein.[1] The definition of "high-quality protein," however, is vague. Consequently, it may be useful in a number of circumstances to consider the ability of a diet to meet the requirements for all of the dietary indispensable amino acids (IAAs), rather than total protein. This approach requires quantifying protein quality in terms of the extent to which ingestion of a certain amount of protein delivers the target intakes of the IAAs. The Food and Agriculture Organization of the United Nations (FAO) developed an approach to quantifying protein quality called the Protein Digestibility–Corrected Amino Acid Score (PDCAAS).[2] The PDCAAS was derived as a means to quantify dietary protein quality on the basis of both the profile and the relative amounts of dietary IAAs in the test protein, corrected for digestibility using a single value for true fecal crude protein digestibility, and expressed relative to a profile of amino acid requirements.[2] A PDCAAS of 1 means that all of the minimal requirements for IAA intake would be met if the amount of the test protein eaten was equivalent to the estimated average requirement (EAR) for protein. For high-quality proteins that have a PDCAAS greater than 1.0, the PDCAAS truncates scores to 1.0. Truncation is used since it was deemed that excess dietary amino acids would not be utilized and therefore should not be included in the PDCAAS values.

The truncation of the PDCAAS at 1.0 eliminates the possibility of distinguishing the relative quality of high-quality dietary proteins. The FAO recently released a document recommending the adoption of a new scoring system to quantify dietary protein quality.[3] The new scoring system, termed the Digestible Indispensable Amino Acid Score (DIAAS), is meant to

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replace the PDCAAS. The conceptual goal of the DIAAS is similar to that of the PDCAAS. With the DIAAS, the quality of a protein is based on the relative digestible content of the IAAs and the amino acid requirement pattern. In contrast to the PDCAAS, the DIAAS is not truncated for a single-source protein, thereby theoretically enabling the ranking of all dietary proteins by their quality. An accurate quantitative ranking of protein quality has great potential for clarifying many aspects of protein nutrition in a general sense, and could be of value specifically in the context of dietary recommendations and the creation of meal plans. The accuracy of the DIAAS is contingent on a variety of factors, including the use of the content and profile of IAAs as the basis for the calculation of quality; the methods for the determination of the protein and amino acid content of the protein source; the accuracy of individual requirement values for IAAs; the normalization of IAA requirements by dividing each value by EAR for protein, and the use of the true ileal digestibility of each IAA in the test protein as the basis for the DIAAS. In light of the many and varied potential applications of the DIAAS, it is pertinent to review the factors underlying the calculation of the DIAAS for individual proteins.

Calculation of the DIAAS

Conceptually, the DIAAS is meant to reflect the percentage of the total daily requirement of the most limiting dietary IAA contained in an amount of protein equivalent to the EAR for total daily protein intake of the test protein.

The equation used to calculate the DIAAS is as follows[1]:

$$\text{DIAAS}(\%) = 100 \times [(\text{mg of digestible dietary IAA in 1 g of the dietary test protein})/(\text{mg of the same amino acid in 1 g of the reference protein})]$$

In practice, the digestible dietary content of each IAA in the test protein is calculated as the content of each amino acid of the protein multiplied by their respective digestibility coefficients, where the digestibility coefficients are based on the disappearance of amino acids from the gastrointestinal tract as measured at the end of the ileum (true ileal digestibility). In the case of lysine and processed food, digestibility is based on that of reactive lysine. A reference ratio of the digestible amino acid content of each IAA in the test protein to the content of the corresponding amino acid in 1 g of the reference protein is calculated, and the lowest value is the DIAAS (expressed as percentage). The amount of each IAA in the reference protein is calculated by dividing the requirement for each IAA by the EAR. Thus, the DIAAS is determined by the most limiting IAA in the test protein in relation to its corresponding content in the reference protein. The amounts and patterns of each IAA in the reference protein reflect the amounts that will meet the daily requirement for each IAA by the ingestion of an amount of that protein equal to the EAR. The calculations are described in detail, along with examples, in a 2013 report by the Food and Agriculture Organization of the World Health Organization.[3]

The reference protein is a theoretical "ideal protein" that reflects the IAA pattern of dietary requirements. To calculate the ratio of the IAAs in the test protein to the corresponding content of the reference protein, it is necessary to express the digestible IAAs in the reference protein in the same units as the test protein. The IAA contents in the test protein are expressed as milligrams per gram of protein, while IAA requirements are expressed as milligrams per kilogram per day. It is therefore necessary to convert the dietary IAA requirements to the units of milligram per gram of reference protein. To calculate the DIAAS, this is accomplished by dividing each requirement value by the EAR. By dividing by the EAR, the ratio of the digestible content of each IAA in the test protein to the corresponding IAA content of the reference protein expresses the fraction of the requirement for the most limiting IAA provided by ingestion of the EAR of the test protein. Multiplying by 100 expresses that value as a percentage.

The DIAAS may be below or above 100%, depending on the quality of the test protein. When the DIAAS is normalized to the EAR for protein, then a value of 110%, for example, means that ingestion of the test protein to meet the EAR would supply 110% of the daily requirement for the most limiting amino acid in the protein, and more than 110% of the requirement for all of the other IAAs in the protein. Similarly, a DIAAS of 90% means that 90% of the daily requirement is provided by the ingestion of 0.66 g/kg/d of the test protein.

The fact that a score is calculated for each IAA and the DIAAS is based on the lowest reference ratio (meaning the most limiting IAA in the protein) complicates quantification of the potential effect of an error in one or more of the factors involved in the calculation of the DIAAS. If a factor affects all of the IAA reference ratios in the test protein to a similar extent, regardless of the most limiting IAA in the test protein, then estimation of the error in that factor directly translates to the DIAAS. On the other hand, if a particular IAA reference ratio is disproportionately affected by an error in a factor, with the consequence that a different IAA becomes the most limiting IAA in the test protein, the

effect on the DIAAS is much more difficult to quantify. Both circumstances arise in the evaluation of the potential errors of each factor.

IAAs and protein quality

The DIAAS is fundamentally based on the amount and profile of digestible IAAs in the test protein in relation to the dietary requirements for each IAA. There is a historical basis for this general approach. Since the 19th century, certain amino acids have been recognized as dietary essential, or indispensable, nutrients, meaning they cannot be produced in the body at all or at a rate sufficient to meet nutritional requirements.[4] These are known as the dietary IAAs. The IAAs are histidine (His), isoleucine (Ile), leucine (Leu), valine (Val), lysine (Lys), threonine (Thr), phenylalanine (Phe), methionine (Met), and tryptophan (Trp). It has also been known for almost 100 years that the requirements for individual IAAs depend on the physiological state, such as growth or maintenance.[4] More recent data indicate that circumstances such as aging, athletic performance, and serious illness may also influence both the amount and profile of the optimal amounts of IAA intake,[5] The basis for the dietary essentiality of the IAAs stems from their role in the process of protein synthesis and the lack of enzymes necessary for their synthesis from other amino acids and compounds. In this section, the role of both the IAAs and the dietary dispensable amino acids (DAAs), or dietary nonessential amino acids, in regulating protein synthesis is examined.

Protein in the body is in a constant state of turnover, meaning that protein mass can only be maintained or increased if proteins that are degraded are replaced by newly synthesized protein. The intake of IAAs is necessary for the synthetic process to equal or exceed the rate of protein breakdown, since a certain fraction of IAAs released in the process of protein breakdown is oxidized, and there is also some irreversible loss of IAAs from the gut and via other routes. In addition to the role of IAAs in protein synthesis, many other roles of amino acids as individual nutrients are becoming appreciated. For example, dietary amino acids serve as precursors for the synthesis of compounds such as neurotransmitters, purines and pyrimidines, and nitric oxide. Nonetheless, the principal metabolic role of dietary IAAs is as precursors for protein synthesis, so it is reasonable that IAA requirements have been based on the impact of IAA ingestion on protein synthesis. Stimulation of protein synthesis is a primary metabolic response to intake of protein/amino acids. Infusion or ingestion of amino acids stimulates whole-body and muscle protein synthesis, thereby positively influencing the balance between synthesis and breakdown (ie, net protein synthesis).[6] Indispensable amino acids are primarily responsible for this response. Ingestion of IAAs alone stimulates muscle protein synthesis as much as a mixture of the same amount of IAAs plus additional DAAs.[7] When IAAs are given alone, the DAAs that are also required for the synthesis of new proteins are derived from more efficient reutilization of endogenous DAAs. In contrast to the stimulatory effect of IAAs on protein synthesis, ingestion of a mixture of DAAs in the profile found in whey protein failed to stimulate muscle protein synthesis.[8]

While it is reasonable to base the scoring of the quality of a protein on the amount and profile of IAAs, the DAAs in a test protein are also of consequence. The importance of dietary DAAs was documented in the early studies of amino acid metabolism. The efficiency of utilization of the IAAs as assessed by nitrogen balance was shown to be enhanced by the amount of DAAs given concurrently.[4] Both IAAs and DAAs are required for protein synthesis, and if IAAs are given in abundance (without DAAs), the resulting stimulation of protein synthesis may deplete the availability of DAAs. The stimulation of protein synthesis by ingestion of IAAs causes plasma DAA concentrations to decrease.[9] This is because both IAAs and DAAs are required to produce new proteins, and if DAAs are not supplied exogenously, the DAAs for increased protein synthesis will be derived entirely from more effective reutilization of endogenous DAAs. If only IAAs are ingested, the availability of certain DAAs could become rate-limiting for protein synthesis. The exact amounts of either total or individual DAAs that are essential components of the diet to maximize the effectiveness of dietary IAAs have not been determined. Agricultural science literature indicates that the ideal composition of feed for the maximum growth and muscle development of farm animals consists of approximately two-thirds amino nitrogen in the form of IAAs,[10] but comparable data for humans are not available. Thus, although there is some (uncertain) need for DAA intake, the prevalence of DAAs in dietary protein is most likely more than adequate to provide ample DAAs when sufficient protein is ingested to meet IAA requirements. Dietary protein contains between 30% and 50% IAAs, which means that the contribution of DAAs to amino acid composition of proteins is more than adequate to meet requirements if the animal literature can be extrapolated to human diets.

There is a relationship between the dose of ingested IAAs and the acute response of protein synthesis and breakdown. At low dietary doses of IAAs, the stimulation of both muscle and whole-body protein synthesis is linearly related to the increase in the availability of IAAs.[6] At higher doses of IAAs, the synthetic response

plateaus; however, there continues to be a gain in net protein balance (ie, synthesis minus breakdown), owing primarily to a suppression of protein breakdown.[11–13] Thus, there is a close linear relationship between the net gain of body protein and the amount of IAAs ingested in a single meal. Over time, however, the effects of ingesting of an increasing amount of IAAs on net gain of body protein reach a plateau because there is a limit to the extent to which protein breakdown can be suppressed. The point at which a plateau is achieved in net gain of body protein depends on a number of factors, including genetic propensity for increasing muscle size, the level of physical activity, total caloric intake, etc. When a plateau is reached despite continued intake of increased amounts of IAAs, protein mass of the body is maintained with a higher rate of protein synthesis and a lower rate of protein breakdown than when IAA intake is lower.[14]

A higher rate of muscle protein turnover due to accelerated protein synthesis will improve muscle quality, presumably by remodeling muscle structure by replacing older, less functional fibers with newly synthesized fibers. Muscle fibers thus remodeled demonstrate improved force and speed of contraction at the single-fiber level,[15] which is reflected in improvements in strength and function that may exceed gains in muscle mass.[16–19] The effect of stimulated protein synthesis in conjunction with a decrease in protein breakdown on muscle function is less certain. Part of the remodeling process involves the breakdown of less-functional proteins, and a reduction in protein breakdown at high levels of IAA intake may limit this response. It may be that IAA intake above that amount required to optimize protein synthesis may increase muscle mass as a consequence of reduction in protein breakdown, without a corresponding improvement in single-fiber function. This issue has not been addressed experimentally.

It is a fundamental underlying assumption of the DIAAS that the quality of a protein is determined entirely by its digestible IAA content. The (unstated) corollary of this assumption is that quality is inversely related to the DAA content of the protein. This is because the amount of DAAs in a protein directly affects the DIAAS by the extent to which the IAAs in the protein are diluted. For example, if one test protein has the exact same profile (relative ratios) of IAAs as another, but the first protein has more DAAs than the second protein, then the DIAAS will be higher in the second protein because the content of IAAs per gram of protein is greater. Consequently, although the DIAAS calculation of quality relies only on the IAA content, understanding the contribution of specific DAAs and total dietary nonessential nitrogen to the response to protein synthesis is directly relevant to the calculation.

Analysis of protein and amino acid content of protein in food sources

Evaluation of protein quality using the DIAAS requires knowledge of the protein content (milligrams of protein per gram of food source) as well as the IAA content and profile. The protein content can be estimated using several approaches, including measurement of the nitrogen content or the summation of the amino acid content. For determining the DIAAS, the FAO[1] did not specifically prescribe how the protein content should be determined but notes that the 1989 consultation[2] recommended that, to calculate the PDCAAS, protein content should be estimated on the basis of nitrogen content. Nitrogen is typically determined using either the Kjeldahl method or the Dumas (total combustion) method, with the typical analytical error (residual standard deviation) generally being less than 2%. The generalized nitrogen-to-protein conversion factor of 6.25 is used to estimate the protein content from the nitrogen content. While food-specific nitrogen-to-protein conversion factors have been derived for a range of foods, they were not recommended when the PDCAAS was developed.[2] An exemplar for calculating the DIAAS is given by the FAO[1] and is based on the generalized factor of 6.25. The choice of the nitrogen-to-protein conversion factor used (generalized factor or food-specific factor) will inevitably impact the digestible IAA ratios and the DIAAS. For example, brown rice has a reported nitrogen-to-protein conversion factor of 5.95,[21] which differs from the generalized factor of 6.25 by 5%. Consequently, a DIAAS value based on the food-specific factor for brown rice will be 5% higher than that based on the generalized factor. In contrast, for foods that have a food-specific nitrogen-to-protein conversion factor higher than 6.25, for example milk proteins,[20] the DIAAS would be lower if food-specific factors were used. Another point to consider is that, while the food-specific factors are likely to more accurately reflect the nitrogen-to-protein ratio of different foods, they do not account for variation in the protein composition that may exist within the same type of food. For example, the protein composition of milk varies seasonally as well as across geographic location and across species and the breed of cow. Moreover, both the processing of milk and the manufacture of milk products involve the fractionation of milk proteins, such that the protein composition of products produced may not necessarily reflect the protein composition of the original milk. For example, whey protein–based foods and ingredients do not contain casein, and casein-based foods do not contain whey proteins. Consequently, a single factor of 6.25 may not accurately represent the nitrogen-to-protein conversion

factor for all milk-based foods. An added potential complication is the presence of nonprotein nitrogen. Nonprotein nitrogen includes compounds such as choline, creatine, purines, pyrimidines, and amino sugars, and the amount of nonprotein nitrogen may also vary across and within food types, particularly less-refined foods and food ingredients. Generally, but not always, nonprotein sources of nitrogen will be present in relatively small amounts.

Protein content of food can also be calculated as the summation of the determined amino acids.[21] The amino acid content can be determined using amino acid analysis and represents the total amino acid content, including amino acids present in protein and peptides as well as any free amino acids present. From a nutritional point of view, determining free, peptide-bound, and protein-bound amino acids collectively is acceptable, given that peptides and proteins are digested and absorbed along with free amino acids in the gastrointestinal tract and therefore utilized equally in the body. One important consideration when attempting to estimate the protein content from amino acid composition data is the molecular weight used to calculate the amount (weight) of each amino acid in the food. Typically, amino acid composition data for foods is calculated using the molecular weight of each of the free amino acids. However, when estimating the protein content, the "in chain" molecular weights must be used. The "in chain" molecular weights are 18 Da less than the free amino acid molecular weights, since they exclude the water added across the peptide bond during hydrolysis. In principle, estimating protein content from the summation of amino acids is sound, as long as all the amino acids can be determined with accuracy. The latter approach has the advantage over the "nitrogen method" described above in that, first, it is unaffected by the presence of nonprotein nitrogen, and second, the protein content can be estimated for the specific food being evaluated for the DIAAS because the amino acid content of the food is determined as part of the DIAAS anyway. Overall, more discussion about the estimation of the protein content of foods is required, and consensus will ultimately need to be reached with respect to the recommended method.

Amino acid analysis is a central component of the DIAAS methodology in that the amino acid content of the food, the test diets, and the ileal digesta is required to determine the true ileal digestible amino acid content of the food. However, amino acid analysis is a technically demanding procedure. Each amino acid possesses a chemically unique side chain, and it is the chemical diversity and relative stability of the side chains that is most problematic for amino acid analysis. Most amino acids are quantified by hydrochloric acid hydrolysis (6 M hydrochloric acid at 110°C for 24 h in an oxygen-free environment) to liberate the amino acids from the protein. The liberated amino acids are then separated and quantified.[22] However, a number of amino acids, particularly methionine, cysteine, and tryptophan, are partially labile in hot acid and are not quantitatively recovered after hydrochloric acid hydrolysis. Consequently, methionine and cysteine are usually quantitatively oxidized to methionine sulfone and cysteic acid, respectively, prior to hydrochloric acid hydrolysis, and the oxidized derivatives are then quantified. For tryptophan, alkali hydrolysis is usually used in place of hydrochloric acid hydrolysis. Other amino acids, such as serine and threonine, also undergo partial degradation (5%–10%) during hydrochloric acid hydrolysis, while valine and isoleucine are difficult to hydrolyze and can also be incompletely recovered after hydrochloric acid hydrolysis for 24 hours. Asparagine and glutamine are completely deamidated to their acid derivatives, aspartic acid, and glutamic acid. For processed food in which amino acids may have undergone chemical modification, eg, Maillard reactions with lysine or oxidation of methionine, cysteine, or tryptophan, specific assays may be needed to accurately determine the amount of unmodified amino acids present.[22] The accuracy of amino acid composition data will inevitably affect the accuracy of digestible IAA ratios.

Approaches to determining individual IAA requirements

Experimental design. Current guidelines promulgated by the FAO[3] for dietary requirements for individual IAAs for individuals 18 years of age and older are based on maintenance levels of intake. There are different values for younger children, but, for the purpose of discussion of the DIAAS, the adult requirements will be highlighted here. The IAA requirements for maintenance are assumed to be the same for all individuals 18 years or older. The amount and pattern of dietary IAAs required for maintenance are based on data derived from experiments that used the general approach of deleting one IAA from an otherwise complete diet and then adding back progressively greater amounts of that amino acid until the selected endpoint indicates that a breakpoint in response has been achieved. The original studies using this approach utilized the point at which zero nitrogen balance was achieved as the endpoint.[23] More recently, isotope tracer studies have been utilized. An extensive series of studies were performed in human subjects in which the endpoint was the inflection point in the curve relating the rate of oxidation of the IAA under study to the intake of that IAA.[24] A modification

of this approach involves measurement of 24-hour balance of the amino acid, using the isotopically measured rate of oxidation as the principal endpoint.[25] The amino acid oxidation approach was modified so that the endpoint reflects the rate of whole-body protein synthesis (the indicator amino acid oxidation [IAAO] methodology).[25] The general principle of the IAAO method is that the oxidation of the indicator amino acid (phenylalanine) is determined at progressively increasing amounts of the specific amino acid under investigation in the setting of excess availability of the other IAAs. As increased amounts of the test amino acid are ingested, the rate of protein synthesis increases correspondingly (reflected by decreasing rates of phenylalanine oxidation). The requirement for the specific test amino acid is considered to be defined by the point at which phenylalanine oxidation plateaus. The inflection point of the oxidation curve is assumed to indicate that there is no further stimulation of protein synthesis,[25] and therefore the requirement for the respective IAA has been met. The IAAO approach uses mixtures of crystalline amino acids rather than complete meals to assess requirement. Whereas this does not exactly reflect the normal form of ingestion, it allows for precise mixtures of amino acids to be tested.

Most of the amino acid oxidation approaches to estimating amino acid requirements underestimate the total anabolic response to amino acid intake because the underlying assumption is that stimulation of protein synthesis is the entire basis for gain in body protein in response to IAA intake. This assumption ignores the fact that net gain of protein in response to protein or IAA intake is a function of the net balance between protein synthesis and breakdown. Both synthesis and breakdown can change in response to protein or amino acid intake,[11–13] and ignoring the reduction in the rate of breakdown at high intakes of protein will result in an underestimation of the true anabolic response.

Another limitation of approaches that have been used to estimate IAA requirements is that it is universally assumed that the amount of one IAA in the diet has no impact on the metabolism or function of other IAAs. It is well established that this assumption is incorrect. For example, increasing the amount of leucine in the diet activates branched-chain ketoacid dehydrogenase, which in turn increases the oxidation of valine and isoleucine in addition to that of leucine. Thus, an increased amount of leucine in the diet may result in decreased availability of isoleucine and valine unless some increase in those amino acids occurs concomitantly.[26] If this interaction is ignored, then the observed response may reflect a decrease in the availability of valine and isoleucine rather than an increased availability of leucine. The relative availability of one IAA can also directly affect the function of other IAAs as a result of competition for the same amino acid transporter. One of the best-established examples is the case of the branched-chain amino acids and the aromatic amino acids, which compete for the same transporter. An increase in the concentration of branched-chain amino acids will decrease uptake of phenylalanine and tryptophan, with physiological consequences such as alteration in the rate of synthesis of serotonin and norepinephrine.[27] The impact of other components of the diet on responses to changes in the availability of a single IAA has also been ignored in the assessment of IAA requirements, even though the effect of the interaction between dietary energy and protein intake on nitrogen balance has been well recognized for more than 100 years.[4] Several issues related to the interaction between protein intake and nonprotein intake could be relevant to the apparent requirement for an individual IAA. For example, it is well established that an increase in carbohydrate intake decreases the oxidation of concurrently ingested IAAs (for example, see Shangraw et al.[28]). The amount of fat intake apparently also has an interactive effect with the response to concurrently ingested protein. For example, whole milk stimulated muscle protein synthesis to a greater extent than the corresponding amount of fat-free milk, despite equivalent contents of protein.[29] In summary, there are many aspects of amino acid nutrition that have yet to be explored, and while the calculation of the DIAAS must rely on currently available recommendations for amino acid requirements, further research to more accurately define amino acid requirements is warranted.

Technical issues related to determination of endpoints to estimate IAA requirements. None of the endpoints that have been used to assess individual IAA requirements are necessarily definitive. Early studies relied on nitrogen balance as the indicator of adequacy of intake of individual IAAs. Nitrogen balance is a crude index that is very difficult to determine accurately enough to define the requirement for a specific amino acid. Data from nitrogen balance studies have been taken into account in the formulation of current recommendations for IAA requirements, but nitrogen balance studies are not considered to be definitive in this regard.

More recent investigations have used stable isotopic tracer techniques to measure the rate of oxidation of various IAAs. In addition to the theoretical issues discussed above, amino acid oxidation approaches are limited by a variety of potential technical problems. Regardless of the specific IAA, the rate of oxidation is difficult to measure accurately. The principal technical difficulty stems from the fact that only a small fraction of total CO_2 production is derived from a single carbon atom in an IAA. As a

result, the enrichment of expired CO_2 is low and difficult to measure accurately. The measurement of total $^{13}CO_2$ excretion is further complicated by the fact that the isotopic enrichment of the expired CO_2, which is expressed as a ratio of $^{13}CO_2$ to $^{12}CO_2$, may be as low as 0.001 or less. The enrichment of CO_2 must then be multiplied by the total rate of CO_2 production (VCO_2) to calculate the rate of $^{13}CO_2$ excretion.[30] Total VCO_2 measured by indirect calorimetry is generally ±5% or more, meaning that the error in the determination of VCO_2 may be much greater than the changes in $^{13}CO_2$ enrichment, which occur at different rates of amino acid oxidation. The difficulty in accurately measuring total $^{13}CO_2$ contributes to the difficulty in determining a definitive "breakpoint" in the amino acid oxidation rate in relation to amino acid intake.

Beyond the issue of accurate measurement of expired $^{13}CO_2$ enrichment, a more fundamental issue with the isotopic methods is that the enrichment of the true precursor for oxidation, namely the intracellular enrichment, must be determined for accurate calculation of amino acid oxidation. Direct measurement of this value is not possible in whole-body experiments in human subjects. When the plasma enrichment is used as the precursor for oxidation, the true rate of oxidation is underestimated.[30] The extent of underestimation depends on the difference between the intracellular and extracellular enrichments of the precursor. It would be expected that higher protein intakes would minimize the difference between intra- and extracellular enrichments of the test amino acid, since a closer equilibrium between intra- and extracellular enrichment would be approached with a higher rate of influx of exogenous amino acid as compared with the intracellular appearance of the unlabeled test amino acid from protein breakdown. This means that, at higher intakes of the test IAA, one would expect the plasma enrichment to underestimate true precursor enrichment to a lesser extent than that during lower intakes. The precursor problem can be minimized in some cases. The best example is the measurement of leucine oxidation.[31] When labeled leucine is used as the tracer, the use of the enrichment of plasma α-ketoisocaproic acid reflects the true precursor enrichment.[31] While the use of alpha ketoisocaproic acid as precursor to measure leucine oxidation improves the accuracy of that measurement, this generally means that values used for measurement of leucine oxidation are not quantitatively comparable with the values obtained from other amino acid oxidation studies, since corresponding surrogates of intracellular enrichment are generally not available.

During the infusion of a ^{13}C-labeled amino acid, not all $^{13}CO_2$ produced at the cellular level appears in the breath.[32] This is because $^{13}CO_2$ can be lost to metabolic pathways via exchange reactions that are not reflective of net flux through that pathway. The extent of these exchange reactions is dependent on the extent to which tracer is lost from the molecule before entering the tricarboxylic acid cycle. If decarboxylation occurs before entry into the tricarboxylic acid cycle, then isotopic retention can be accounted for by performing a partner experiment in which retention of the label from the bicarbonate pool is determined, and correction for that amount of retention is made. The correction factor is not trivial (about 20%), and is potentially variable, so failure to account for this can result in a significant quantitative error. This correction is usually made in amino acid oxidation studies. Of even greater concern, if the label enters the tricarboxylic acid cycle before decarboxylation, the potential for isotopic exchange is much greater and highly dependent on the metabolic state. Exchange within the tricarboxylic acid cycle can be accounted for by performing a partner experiment using ^{13}C-labeled acetate, which reflects the isotopic exchange of acetyl coenzyme A within the tricarboxylic acid cycle.[32] The position of the label in the test amino acid must be taken into account, as there is an approximate 50% difference in recovery of labeled $^{13}CO_2$ when 1-^{13}C-acetate is used as compared with 1,2-$^{13}C_2$-acetate.[32] It is unclear whether this issue has been addressed in any of the amino acid tracer experiments used to determine IAA requirements.

The natural enrichment of carbon in different foods varies significantly in relation to the magnitude of $^{13}CO_2$ excretion that results from the infusion of a ^{13}C-labeled amino acid.[33] This issue has been universally ignored in all of the tracer studies measuring tracer recovery as $^{13}CO_2$ for the purpose of estimating amino acid requirements. The magnitude of variation of naturally occurring $^{13}CO_2$ is significant in relation to the amount of $^{13}CO_2$ produced during a tracer experiment designed to quantify amino acid oxidation during a meal or during substrate intake. It is impossible to know the impact of this issue on the results of the tracer studies defining amino acid requirements without knowledge of the naturally occurring ^{13}C enrichment of the dietary intake. The only way to account for naturally occurring ^{13}C in a test diet is to run a partner experiment with the same meals, but without any tracers. This approach has never been taken in the tracer experiments defining IAA requirements.

The limitations of amino acid balance methods based on stable isotope tracer methodology to derive estimates of IAA requirements have been recognized by proponents of the approach.[34] Numerous alternative approaches are now available to accurately measure changes in body composition over time, such as dual energy X-ray absorptiometry, and a variety of

functional tests have been validated that are sufficiently sensitive in at least certain groups of individuals, such as older individuals, to enable quantitative assessment of the response to various levels of amino acid intake.[17–19] Published requirement levels could now be reassessed, utilizing established measures of body composition and functional outcome.

Validity of IAA requirements

The values for IAA requirements presented in the document describing the DIAAS[2] are the best estimates currently available. However, the preceding discussion of the methods used leads to the logical conclusion that these cannot be considered definitive. This perspective is supported by consideration of the total IAA requirement values in relation to requirements for total protein intake. The sum of the requirements for all IAA requirements as defined by the FAO is 0.184 g/kg/d, and the EAR for total protein intake is 0.66 g/kg/d.[1] Thus, only 28% (ie, [0.184 g/kg/d ÷ 0.66 g/kg/d] × 100) of total protein intake needs to be IAAs. Accordingly, more than 70% of the average required protein intake is deemed to comprise DAAs. Furthermore, that value is close to 80% if IAA requirements are considered in relation to the Recommended Dietary Allowance (RDA) of protein (0.8 g of protein per kilogram body weight per day).[1] The RDA is meant to represent the amount of protein intake adequate to meet the needs of 98% of normal individuals. It is known that some amount of DAAs is required to optimize the response to dietary IAA intake, but it is unlikely that more than 70% of total amino acid intake should be in the form of DAAs. Rather, it seems likely that the discrepancy between the EAR and the requirement for total IAAs is due at least in part to the underestimation of the optimal level of intake of IAAs. An alternative explanation is that the EAR is too high, but there are ample data documenting that not only is this not the case, but that the EAR may in fact be too low.[35] It is also possible that total protein requirements reflect the amount of protein that must be eaten to meet IAA requirements, and the apparent requirement for a large amount of DAAs simply reflects the normal composition of dietary protein. Since most dietary proteins contain between 35% and 50% IAAs, more DAAs than IAAs will normally be consumed. However, the normal composition of dietary protein cannot fully explain the difference between the IAA requirements and the total protein requirements set forth by the FAO, since the theoretical "ideal" protein that exactly meets all IAA and DAA requirements contains only 28% IAAs and 72% DAAs. Thus, in practice, if all IAA requirements are met exactly by the EAR of a normal dietary protein, less than the required amount of DAAs would be ingested. Whereas the requirement for dietary DAAs is uncertain, it is unlikely to be greater than that supplied by the ingestion of the EAR of normal dietary proteins necessary to meet IAA requirements. Since it is unlikely that protein requirements are dictated by the need for DAAs, it follows that IAA requirements are likely underestimated.

There are data related to physiological function to support the contention that current estimates of IAA requirements are too low. The amounts of endogenous threonine and lysine lost daily via the digestive tract of the adult human have been calculated.[36] To maintain gut protein mass and gut functions, any amino acid lost from the body must be replaced and therefore constitutes part of the daily requirement for dietary amino acids. The predicted total loss of gut-endogenous lysine accounts for almost three-fourths of the FAO's estimated lysine requirement,[1] while the highest predicted loss of threonine from the gut exceeds the FAO's estimated threonine requirement value and is very close in magnitude to more recently published higher values for threonine requirement.[37] Dietary IAA intake must replace not only the loss of IAAs via the digestive tract but also the oxidation of IAAs released by body protein breakdown. The extent of oxidation of IAAs varies according to the physiological circumstance but usually constitutes about 20% or more of flux.[38] Thus, the requirement for IAAs clearly must be greater than the amount lost via the gut alone. This provides a further indication that published estimates of at least some of the IAA requirements are too low.

Indispensable amino acid requirements are a fundamental basis of the calculation of dietary protein quality by the DIAAS. The content of each IAA in the test protein is compared with the corresponding amino acid value of the reference protein, which is in turn calculated from the requirement for that IAA, the true ileal digestibility of that amino acid in the test protein, and the EAR. The accuracy of the DIAAS is thus impacted directly by IAA requirements. If all of the digestible IAA requirements are proportionately too low, then the DIAAS will overestimate the extent to which ingestion of the EAR of the test protein will meet IAA requirements. The relative ranking of protein quality would not be affected in this circumstance. If, on the other hand, some IAA requirements are in error to a greater or lesser extent than others, thereby changing the profile of requirements, not only would the absolute DIAAS be affected, but so too might the relative rankings of protein quality, depending on the limiting IAA in the individual test proteins. It is therefore relevant to consider in more depth the profile of IAA requirements in addition to the absolute levels of requirements for individual IAAs.

Impact of the IAA/DAA ratio in the reference protein

As described above, the calculation of the DIAAS involves dividing each IAA requirement by the EAR to derive the amino acid scoring pattern in milligrams per gram of protein. Implicit in this calculation is the assumption that the EAR is for completely balanced, or "ideal" protein, meaning that ingestion of the EAR of the reference protein would exactly meet all IAA and DAA requirements (100% of the reference protein). Consequently, the profile of the IAAs in the reference protein must be the same as the profile of IAA requirements, and there must be an ideal ratio of total IAAs to total DAAs. Deviations from either the "ideal" IAA/DAA ratio in the reference protein or the profile of IAAs will directly impact the calculated DIAAS. The requirement for total IAAs is 0.184 g/kg/d, and since the EAR is 0.66 g/kg/d, the amount of recommended DAA intake is $0.66 - 0.184 = 0.476$ g/kg/d. This means that the IAA/DAA ratio in the "ideal" reference protein is 0.38. If the optimal IAA/DAA ratio in the reference protein is actually lower than 0.38, an intake less than the EAR of a test protein would be needed to meet IAA requirements (the DIAAS would be higher than if calculated using the "ideal" protein as the reference protein). Similarly, if the current requirement value for a single IAA is less than the true value, ingestion of less test protein would be required to meet the target intakes of all IAAs if the limiting IAA in the test protein corresponded to the IAA that is underrepresented in the reference protein (ie, a higher DIAAS). Conversely, a higher IAA/DAA ratio in the reference protein will result in a lower DIAAS because more of the test protein would be needed to meet total IAA requirements. Some numerical examples of the impact of differences in the IAA/DAA ratio are shown in Table 1.

Changing the IAA/DAA ratio in the reference protein uniformly affects the DIAAS in all cases. In contrast, a different profile of IAA requirements can have a variable effect on the DIAAS, depending on the limiting IAA in the test protein. For example, a different profile of IAA requirements was developed using the IAAO methodology.[25] Not only do the absolute values of requirements developed by the IAAO methodology differ from those used by the FAO, but the profiles differ as well. The IAA reference ratios differed correspondingly. Depending on which set of reference ratios is used, a different DIAAS of a dietary test protein will be calculated. The DIAAS will be affected to a different extent if the limiting amino acid in the test protein is affected by the choice of reference ratios that is used. For example, the DIAAS would change from 75 to 55 for cooked rice and from 141 to 91 for milk protein concentrate, depending on which profile is used for the reference protein.

Table 1 **Digestible Indispensable Amino Acid (DIAA) reference ratios and Digestible Indispensable Amino Acid Score (DIAAS) for whole-milk powder and cooked rice,[1] calculated using a recommended amino acid scoring pattern based on an ideal protein that differs in the ratio of total indispensable amino acids (IAAs) to total dispensable amino acids (DAAs)**

	IAA/DAA ratio in the reference protein				
Whole-milk powder[a]	28:72[b]	40:60	45:55	50:50	55:45
DIAA reference ratio[c]					
Histidine	211	148	131	118	107
Isoleucine	191	134	119	107	98
Leucine	198	139	123	111	101
Lysine	222	156	139	125	113
Methionine/cysteine	141	99	88	79	72
Phenylalanine/tyrosine	328	230	204	184	167
Threonine	213	149	133	119	109
Tryptophan	272	191	170	153	139
Valine	170	119	106	95	87
DIAAS	141	99	88	79	72
IAA/DAA ratio in the reference protein					
Rice[a]	28:72[b]	40:60	45:55	50:50	55:45
DIAA reference ratio[c]					
Histidine	143	100	89	80	73
Isoleucine	116	81	72	65	59
Leucine	110	77	69	62	56
Lysine	75	52	46	42	38
Methionine/cysteine	124	87	77	69	63
Phenylalanine/tyrosine	224	157	140	126	114
Threonine	103	72	64	58	53
Tryptophan	260	182	162	146	132
Valine	101	71	63	57	52
DIAAS	75	52	46	42	38

[a]The true ileal digestible amino acid content of whole-milk powder and cooked rice was obtained from Rutherfurd et al. (2015).[61]
[b]The IAA/DAA ratio of 28:72 reflects that published by the FAO.[3]
[c]Calculated as follows: DIAA reference ratio = (mg of digestible DIAA in 1 g of the dietary protein) × 100 (mg of the same DIAA in 1 g of the reference protein), where the amino acid profile of the reference protein is the amino acid requirement pattern for adults (>18 y) (g/kg protein), which was in turn based on the amino acid requirements (g/kg/d) multiplied by the amount of an ideal protein that would need to be consumed to meet the amino acid requirements, assuming different ratios of IAA/DAA in the reference protein.

In this section, the importance of the relationship between the IAAs and DAAs in the reference protein for calculation of the DIAAS of a test protein was examined. There are few data to assess whether the currently assumed relationship between IAAs and DAAs in the reference protein is ideal. The fact that the IAA/DAA ratio is lower for the reference protein than that commonly found for high-quality proteins suggests that data directly addressing this issue would be beneficial.

Profile of IAA requirements

The profile of amino acids of a test protein can significantly affect the physiological response to ingestion of the protein, independent of the amount of protein ingested. Indispensable amino acid requirements are meant to define minimal acceptable levels of intake and therefore do not account for any unique metabolic roles of individual amino acids beyond their contribution to body protein as precursors to protein synthesis. The response of protein synthesis in older individuals provides a good example of the shortcoming of this approach. "Anabolic resistance" of aging refers to the fact that intake of a given amount of protein or amino acids stimulates protein synthesis less in an older individual than in a young individual.[38] Several experiments have demonstrated that anabolic resistance of aging can be overcome, to at least some extent, by altering the profile of an IAA mixture without changing the total amount of IAAs ingested. These were based on the regulatory role of leucine as an activator of protein synthesis through a mechanism independent of its role as a precursor for protein synthesis.[39] The resulting profile of IAAs differs markedly from the profile of the minimal requirements for IAAs. In a cohort of older individuals, the response of muscle protein synthesis to ingestion of 6.7 g of a high-leucine mixture was compared with the response to ingestion of 6.7 g of IAAs in the profile of whey protein.[38] The IAAs in the profile found in whey protein effectively stimulated a modest anabolic response, but it was significantly less than the corresponding response in younger individuals.[38] When the same amount of IAAs was reformulated to consist of 40% leucine and appropriate proportions of the other IAAs to normalize the inward transport rates in relation to the IAA composition of muscle, the magnitude of stimulation of muscle protein synthesis was approximately doubled.[26] The difference in response to different profiles of IAAs was particularly striking because of the relatively high leucine content of whey protein as compared with other proteins. These results are relevant to current IAA requirements, since the altered profile of IAAs was much more important in older individuals than in younger subjects,[26] whereas the requirements do not distinguish between young (ie, 18 years) and older individuals. In addition, these results illustrate that a profile of IAAs that deviates widely from the profile of the current IAA requirements may stimulate protein synthesis more effectively than a high-quality protein that closely matches IAA requirements. This experiment also underscored the interactive effects of dietary IAAs, since ingestion of leucine alone had no effect on muscle protein synthesis.

The importance of the profile of IAAs in terms of stimulation of protein synthesis suggests that IAA requirements may be more reasonably defined in terms of the profile of IAAs and in relation to specific physiological states. While this may seem an inherently impossible task, given all of the possible permutations of IAA profiles, the use of preliminary physiological experiments can narrow the number of possible profiles to be tested. The approach described here for defining the optimal profile to stimulate muscle protein synthesis in older individuals was based on results from studies utilizing stable isotope tracer methodology and a 3-pool model of muscle protein metabolism in human subjects that enabled quantification of muscle protein synthesis, breakdown, and amino acid transmembrane transport of all IAAs within a 2- to 3-hour framework.[40] This or a similar approach could be used to re-examine IAA requirements in a manner that accounts for both the amounts of individual IAAs as well as the profile of the mixture of IAAs.

Implications of profile of IAA requirements for calculation of the DIAAS

Differences in the relative values (profile) of IAA requirements have a major impact on both the absolute DIAAS as well as the relative ranking of proteins according to quality. This is because the DIAAS is based on the protein intake needed to meet the required amount of the most limiting IAA, and an alteration in the profile of requirements could change the limiting IAA in one protein as compared with that in a different protein. Previous research defining IAA requirements has focused only on isolated changes in the availability of one IAA. It is clear that investigations of the requirements for individual IAAs in the context of defined profiles of IAAs are warranted.

Use of the EAR for protein to calculate the DIAAS

The IAA requirements must be normalized by a rate of intake of protein so that a reference ratio can be calculated. The FAO consultation group elected to use the EAR for protein for this purpose.[3] The EAR for protein published in *the Dietary Reference Intakes for Energy, Carbohydrates, Fiber, Fat, Protein and Amino Acids*[1] is 0.66 g/kg/d and represents the minimal intake of nitrogen that can enable the attainment of zero nitrogen balance (N-balance) in 50% of the population.[1] In relation to the DIAAS, there are two principal issues related to the selection of the EAR: Is it reasonable to normalize IAA requirements by dividing by an index of protein requirement; and, assuming that it is reasonable to normalize protein quality by an estimation of protein requirement, is the EAR a good reflection of protein requirements?

Normalizing IAA requirements by an index of total protein requirement has the conceptual limitation that it would be extremely unusual to consume the entire recommended intake of dietary protein in the form of a single protein. On the other hand, the use of the EAR has the advantage of normalizing the IAA content of a test protein to a standard reference with physiological meaning. When considered from this perspective, it is as reasonable to use an index of protein requirement as any other expression of protein intake. The more difficult question is whether the EAR is the most appropriate expression of protein requirement.

There are two aspects to the question of whether the EAR is an appropriate expression of protein requirement. The EAR reflects the dietary intake necessary to avoid a deficiency in the nutrient in 50% of the population. Thus, in the case of the EAR for protein, approximately half of the population would be failing to meet dietary requirements for protein intake. Dietary recommendations are more commonly expressed in terms of the RDA, which is based on the EAR + 2 standard deviations. Thus, the RDA is meant to reflect a safe level of intake that will meet requirements in almost all of the population. Furthermore, there is considerable evidence that optimal protein intake in the context of a complete diet is considerably higher than the RDA. The acceptable macronutrient distribution range, or AMDR, also published in the *Dietary Reference Intakes for Energy, Carbohydrates, Fiber, Fat, Protein and Amino Acids*[1], recommends that protein constitutes 10% to 35% of caloric intake, which corresponds to a protein intake of 1.0 g to 3.5 g/kg/d for an adult with an average level of activity.[1] Consequently, there is not a unique value for protein requirement, and the use of the minimal value (ie, the EAR) could be questioned on the basis that it reflects an insufficient intake in so many individuals.

The choice of which expression of recommended dietary protein intake is used to normalize IAA requirements is complicated by the apparent incongruity between IAA requirements and protein requirements discussed above. Consequently, even when the EAR is used to normalize IAA requirements, DIAAS scores of high-quality proteins indicate that 140% or more of IAA requirements will be met by ingestion of the test protein at 0.66 g/kg/d. This would mean that, if the protein requirement was expressed in terms of the amount of intake needed to satisfy IAA requirements, the EAR for protein would be less than 0.5 g/kg/d if the diet comprises high-quality proteins. Use of either the RDA or the AMDR, instead of the EAR, would mean that dietary IAA requirements could be met even with low-quality proteins. However, there is no evidence that a diet comprising high-quality proteins would result in a lower EAR. In fact, the *Dietary Reference Intakes for Energy, Carbohydrates, Fiber, Fat, Protein and Amino Acids*[1] specifically states that the EAR is based on the ingestion of high-quality proteins. Thus, use of the EAR minimizes the potential confusion between protein requirements and IAA requirements.

Despite the uncertainty about which expression of protein requirements should be used to normalize IAA requirements, the relative quality ranking of proteins is not affected. The value for EAR (or any other expression of protein requirements) is a constant in the calculation of the DIAAS for all proteins. The DIAAS could be calculated for any arbitrary amount of protein ingested, provided that the number is a constant throughout the calculation of the DIAAS for all test proteins.

True ileal digestibility of IAAs

Calculation of the DIAAS involves correction of the IAA content of the test protein for the true ileal digestibility of each IAA. The FAO recommends that true ileal amino acid digestibility, rather than true fecal nitrogen digestibility, be used to correct for the digestibility of amino acids.[3] This is one of the key differences between the PDCAAS and the DIAAS. Determining digestibility at the ileal level is fundamentally superior to determining it at the fecal level because, in the hindgut, there is little absorption of amino acids but an abundance of microflora that will digest and utilize undigested protein, peptides, or amino acids exiting the small intestine.[41,42] Moreover, the hindgut microflora can also synthesize amino acids.[41,42] Consequently, the catabolism and synthesis of amino acids by the hindgut microflora will inevitably confound fecal measurements of protein or amino acid digestibility. Furthermore, fecal protein is largely bacterial protein, the amino acid composition of which bears no resemblance to the undigested dietary protein leaving the ileum. Determining digestibility at the end of the small intestine (ileal digestibility) overcomes these problems.

In contrast to the PDCAAS, the DIAAS is based on the digestibility of individual amino acids, rather than the digestibility of crude protein, which is in turn based on nitrogen digestibility. The latter distinction is important since true ileal amino acid digestibility can vary markedly across amino acids, even within the same protein source. As an example, in a recent study examining the true ileal amino acid digestibility of foods consumed in India, true ileal amino acid digestibility differed by more than 20% across the dietary IAAs for many foods and food ingredients examined.[42] Even for highly digestible protein sources, the range in true ileal amino acid digestibility across IAAs within a protein source can be significant.[43]

Ideally, true ileal amino acid digestibility for use in the calculation of DIAAS should be determined in human subjects. However, for logistical and ethical reasons, this is not routinely possible. As a result, the FAO[1] has recommended that, in the absence of true ileal amino acid digestibility data derived using humans, true ileal amino acid digestibility can be determined using the growing pig (the preferred animal model) or the growing rat.

Determining true ileal amino acid digestibility involves feeding a test protein to test subjects/animals and then sampling ileal digesta from the terminal ileum (end of the small intestine). The amino acid contents of the test diet and ileal digesta are then determined. When the collection of digesta is less than complete and analysis is based on a sample of digesta, indigestible markers must be used and ileal amino acids normalized for food dry matter intake (ie, expressed as grams per kilogram of dry matter intake) based on the relative concentration of the indigestible marker in the diet and digesta. Digestibility is then calculated as the difference between the dietary amino acids and the ileal amino acids,[44,45] after correction for the endogenous ileal amino acids, and expressed as a percentage of the amino acid contents of the test diet.

Endogenous ileal amino acid losses represent the nondietary protein present in the gastrointestinal tract and comprise gut protein secretions such as mucins, digestive enzymes, bile salts, and serum albumin as well as sloughed epithelial cells and microbial cells (nondietary, but not strictly endogenous). While a significant amount (≈80%) of the endogenous proteins are digested and reabsorbed by the end of the small intestine,[46] ileal digesta will inevitably comprise protein, peptides, and amino acids from both undigested dietary protein as well as from endogenous proteins. When ileal amino acid digestibility is determined without correcting the total ileal amino acid flows for the endogenous ileal amino acid flows, then digestibility is referred to as apparent ileal amino acid digestibility, while true ileal amino acid digestibility values are determined after correction for the endogenous ileal amino acid flows.

For determining true ileal amino acid digestibility in humans, ileal digesta can be sampled directly using ileostomates in whom the large intestine has been surgically removed in response to an underlying disease, for example, colon cancer.[47] For ileostomates, digesta are collected directly into a colostomy bag, which is attached to the externalized terminal ileum, or stoma. The advantage of using ileostomates is that digesta collection is straightforward. However, the ileostomate gastrointestinal tract cannot be considered normal, and ileal digesta collected from ileostomates may not adequately represent terminal ileal digesta in the normal human gastrointestinal tract. Alternatively, it is possible to collect digesta from normal human subjects by means of a naso-ileal tube.[48,49] The naso-ileal tube is a double-lumen tube that is passed through the nasal cavity, esophagus, stomach, and small intestine down into the terminal ileum. The advantage of naso-ileal sampling is that it can be used with normal human subjects. However, the disadvantages are that subjects must generally undergo hospitalization for several days and, since only small amounts of digesta can pass through the tube and the method relies on multiple markers, the extent to which the sample of digesta represents the total digesta is questionable. Moreover, the material fed to the subjects must be fine, as tube blockage with food particles can be a problem.

Animal models, such as the growing pig and the growing rat, can be used in place of human subjects for determining true ileal amino acid digestibility. If growing pigs are used, then ileal digesta can be collected using a variety of techniques. The most straightforward and commonly used methods include the slaughter technique and ileal cannulation techniques. With the slaughter technique, the terminal ileum is dissected from an anesthetized pig (which is then immediately euthanized) or from the pig immediately post mortem. For the ileal cannulation approach, several techniques have been developed, including simple T-cannulation, postvalve T-cecum cannulation, re-entrant cannulation, and steered ileal-cecal valve cannulation. Each of these techniques has advantages and disadvantages, which have been thoroughly reviewed by others.[50,51] However, the main advantages and disadvantages center around the ability to collect digesta quantitatively and the impact of cannulation on normal gut function. The preferred techniques in practice are simple T-cannulation and postvalve T-cecum cannulation, which, along with the slaughter method, rely upon the use of an indigestible dietary marker.

The simplest cannulation technique involves simple T-cannulation in which a flanged wide-bore cannula (in the shape of a T) is surgically implanted approximately 10 cm anterior to the ileocecal junction. The cannula protrudes through the abdominal wall and is therefore externalized. The external portion of the cannula is normally capped. However, the cap can be removed and a plastic bag attached to the cannula outlet when digesta are to be collected. True ileal amino acid digestibility values determined in pigs using either the simple T-cannulation method or the slaughter method for digesta collection have been shown to be similar.[52]

The main concerns with ileal measurement of amino acid digestibility revolve around how well digesta samples reflect the total digesta, the adequacy of the indigestible marker compound, the correction of the total

amino acid flow for the nondietary amino acid flow, and any effects small intestinal bacteria may have on digestibility. However, a considerable amount of work has been devoted over the years to developing and testing such animal-based assays, and these have been shown to be particularly accurate for predicting amino acid absorption in the target animal species.[53,54] The digestion of protein between the mouth and terminal ileum in the growing pig is similar to that in the adult human,[55] and where direct comparison has been made, true ileal amino acid digestibility values for foods are very similar between pigs and humans.[42,48,56]

Another important facet of the DIAAS is that it is recognized that, for some processed dietary protein sources, amino acids may have undergone structural changes and, although the altered amino acids may be absorbed, they are not available for protein synthesis.[57,58] In these cases, conventionally determined true ileal amino acid digestibility values are not accurate indications of availability. This is an important point because most foods consumed by humans undergo some sort of processing, whether it be processing at the manufacturing level or through cooking in the home. In addition, lysine, which is first limiting in cereals, and methionine, which is first limiting in legumes, are both highly susceptible to chemical modification during processing. For lysine, which can undergo Maillard reactions, early Maillard products are not nutritionally available but can interfere with the determination of lysine during amino acid analysis, leading to an overestimation of lysine in both diets and digesta for heat-processed protein sources.[59] On the other hand, methionine can be oxidized, and the oxidized derivatives are either poorly utilized or not nutritionally available at all.[60] Consequently, for lysine and methionine in processed protein sources, alternative analytical strategies are required that permit the determination of the unmodified amino acids alone in diets and ileal digesta. When such alternative analytical strategies are used, then the true ileal digestibility of the structurally unaltered amino acid availability, rather than true ileal amino acid digestibility, is determined.

Determination of the individual values of digestibility is relevant to the accurate determination of both the amount and the profile of IAA absorbed from a protein. In contrast to the situation with the EAR and the IAA requirements, in which case errors in the total amount of protein or IAAs required will change the absolute DIAAS but should not affect relative rankings of protein quality, errors in the determination of the digestibility of individual IAAs can impact both the absolute DIAAS and the relative ranking of test proteins. The absolute DIAAS will be affected because, if digestibility is low, then the actual delivery of IAAs is less than that reflected by the amount of test protein ingested. The relative rankings could be affected because the profile of the digestible IAAs from the digestion of a protein may differ from the profile of the test protein. When the profile of the absorbed IAAs is altered, the limiting amino acid of some, but not all, proteins will be affected. In this circumstance, not accounting for true ileal digestibility would spuriously affect the relative rankings of protein quality. Comparison of the DIAAS calculated using true ileal nitrogen digestibility as opposed to true ileal amino acid digestibility shows a variable magnitude of effect if the profile of amino acid digestibility is not measured directly. For example, the DIAAS for chickpeas is 88.4% (with valine being the limiting amino acid) when calculated using true ileal nitrogen digestibility and 84.8% (with leucine as the limiting amino acid) when calculated using true ileal amino acid digestibility (4.4% difference). On the other hand, the DIAAS for mung beans is 73.2% (with threonine being the limiting amino acid) when calculated using true ileal nitrogen digestibility and 51.7% (with leucine being the limiting amino acid) when calculated using true ileal amino acid digestibility (29.4% difference).

There is no way to predict a priori the importance of measuring true ileal amino acid digestibility directly as opposed to grouping all amino acids together and measuring only true ileal nitrogen digestibility. In some, but not all, circumstances, relying solely on true ileal nitrogen digestibility will result in a significant error in the calculation of the DIAAS. Unfortunately, ileal digestibility data are available for only some proteins. The FAO recommends using fecal protein digestibility values when true ileal digestibility values are not available, although this could affect quality scores and rankings. Clearly, more ileal digestibility data are needed to fully realize the advantage of using true ileal amino acid digestibility in the calculation of the DIAAS.

CONCLUSION

Indispensable amino acids are the crucial components of dietary protein that are primarily responsible for the stimulation of protein synthesis. Consumption of an adequate amount of protein to meet the requirements for all of the IAAs is therefore central to achieving the goals of a complete diet. It would thus be useful to quantitatively score dietary proteins in terms of their ability to deliver the dietary requirements for IAAs. The DIAAS was developed for this purpose. Conceptually, the DIAAS is meant to reflect the percentage of the total daily requirement of the most limiting dietary IAA contained in an amount of protein equivalent to the EAR for total daily protein intake of the test protein. The

calculation of the DIAAS is based on the content and profile of the IAAs in the test protein in relation to their respective requirements, and on the extent of digestion of each IAA in the test protein in the ileum. The score and its underlying components are sound conceptually, and the component values currently used in the calculation of the DIAAS are, in the consensus opinion of experts in this field,[3] based on the best data currently available. This, however, does not mean that the values cannot be improved. The approaches used to derive IAA requirements are fraught with theoretical and technical limitations. Improving the accuracy of these values is important for the assessment of dietary protein quality by means of the DIAAS.

It is likely that the currently defined requirements for the IAAs are low in relation to the requirement for total dietary protein. Furthermore, not only would the DIAAS be improved by refinement of individual IAA requirements, it would be advantageous to evaluate IAA requirements in terms of the profiles of the IAAs. A single incorrect value of an IAA requirement would change the profile of IAAs in the reference protein, which in turn could affect which amino acid in the test protein is deemed to be limiting. Thus, any alteration in the profile of IAAs in the reference protein could potentially affect not only the absolute DIAAS but also the relative rankings of test proteins. Consequently, it is reasonable to approach the assessment of refining IAA requirements by also considering differing profiles of IAAs, rather than exclusively considering the requirements of each IAA independently. Evaluating IAA requirements in relation to the profile in the test protein could account for interactions between dietary IAAs that are not reflected by individual requirements.

It is not inherently obvious that the optimal amount of DAAs contained in the reference protein is relevant to the calculation of the DIAAS, but the amount of DAAs in the reference protein determines the relationship between the requirement for IAAs and the requirement for total protein intake (ie, the EAR). The requirements for both IAAs and total protein are components of the calculation of the DIAAS. Studies addressing the role of the DAAs in modifying the response to IAA intake would therefore provide crucial information that would improve the accuracy of the DIAAS. The DIAAS is inversely related to the amount of DAAs in a test protein, and directly related to the DAA content of the reference protein.

Most of the parameters used to evaluate protein and IAA requirements, such as nitrogen balance and isotopic studies of IAA oxidation, are indirect measures of adequacy. There is value in measurements of nitrogen balance, in part because of the volumes of studies that have been performed using this methodology. On the other hand, the limitations of the nitrogen balance technique are well documented. Valuable information has been obtained from isotopic studies of IAA oxidation, but greater attention to the principles and practice of tracer methodology would enhance further tracer studies to quantify IAA requirements. Importantly, methods now exist for evaluating outcomes using measures of lean body mass and physical function that could also be used to evaluate requirements. Such approaches could be used not only to assess requirements for total protein and individual IAAs but also to develop different profiles of IAAs and proteins in the context of complete meals.

The extent of digestion of individual IAAs in a test protein is important when determining the DIAAS. Inclusion of true ileal digestibility of individual IAAs in a test protein distinguishes the DIAAS from previous scoring systems such as the PDCAAS. The techniques for determining true ileal amino acid digestibility are well established, but obtaining additional data on the ileal digestibility of IAAs and on lysine availability in various proteins is imperative. Optimally, such data would be generated from human studies.

Finally, values for protein and IAA requirements and digestibility in different physiological states would be valuable. Current recommendations do not distinguish adult populations above the age of 18 years, yet there is ample evidence that requirements change in many different physiological and pathophysiological circumstances.

When all of the limitations of the components of the DIAAS are considered, it is clear that the current approach for calculation is the best available, though not likely to be definitive. Research to further define the components of the DIAAS should target the issues cited above that affect the relative scores of different test proteins. Most prominently, individual IAA requirements have the greatest potential for affecting not only absolute scores but also relative scores of different test proteins. Consequently, it would be reasonable to focus attention on further assessment of individual IAA requirements, including the impact of differing profiles of IAAs. It would also be reasonable to evaluate endpoints that directly reflect the physiological impact of dietary IAAs, such as body composition.

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